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Survival and Multiplication of *Vibrio cholerae* in the Upper Bowel of Infant Mice

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The survival and multiplication of *Vibrio cholerae* strains of varying virulence in the upper bowel of orally challenged infant mice early in infection has been examined. Analysis of changes in the apparent specific activity (counts per minute per colony-forming unit) of the cell population after 4 h compared with the inoculum indicated that strain CA401 established a viable, multiplying cell population, whereas strains VB12 (a rough variant) and 569B were subject to host bactericidal and bacteriolytic mechanisms. An analysis of parameters which may affect the specific activity is included. We have defined the infective potential of the strains in terms of the changes in specific activity. The relative infective potentials are CA401 > 569B > VB12.

In human cholera, infecting *Vibrio cholerae* are confined to the mucosa and lumen of the intestinal tract (14). The organisms elaborate a protein enterotoxin which mediates the net fluid secretion manifested as acute diarrhea (8). Fluid loss originates entirely in the small intestines and primarily in the jejeunum (1). To instigate this diarrheal response, the vibrios presumably colonize the small intestine mucosal surface (13, 22, 23, 27). It is likely that specific bacterial functions are necessary to establish a multiplying *V. cholerae* population in the presence of active host clearing mechanisms such as peristalsis (8), mucus flow (11), and intestinal bactericidal phenomena (11, 12, 25). However, characterization of the bacterial factors which contribute to establishment and maintenance of an infection is largely incomplete.

Similarly, in experimental infections of infant mice, the small intestine appears to be the critical colonization site (6, 16, 20). Furthermore, critical events which influence the course of the disease process appear to occur early in infection (4). In this report, we evaluate the survival and multiplication potential of several strains of *V. cholerae* in the upper bowel of orally inoculated infant mice soon after challenge.

The strains used have been shown previously to differ in disease-inducing capacity for infant mice (3). Strain CA401 is an infant mouse-virulent human isolate (18). VB12 is a non-diarrhea-inducing rough CA401 variant. Strain 569B is a highly toxinogenic laboratory strain (10) which evokes a diarrheal response in infant mice only at high dose levels (3). The goal of this study was to determine whether the differences in virulence among these strains could be correlated with differences in ability to multiply in the upper bowel and to evade normal host clearance mechanisms (mechanical or antibacterial). The methodology used was based on previous studies by Rowley et al., who used ^{32}P-labeled *V. cholerae* in infant mice in which the ratio of radioactivity to colony-forming units (CFU) (specific activity) of an inoculum was compared with the specific activity at varying times postchallenge (6, 20). A change in specific activity with time can be interpreted as an indication of alterations in the distribution of label in terms of viable versus nonviable cells and cell-associated versus non-cell-associated materials. An analysis of events which may contribute to an increase or decrease in specific activity is included in this paper.

MATERIALS AND METHODS

Bacterial cultures. The source of cultures has been given previously (3). Stocks were maintained in lyophilized form or on meat extract agar at 4°C.

Infant mice. CFW mice weighing 3.0 ± 0.5 g (about 5 to 7 days old) were obtained from our breeding colony at the University of Texas Animal Resources Center. Litters were removed from breeders and pooled for experiments; infants were not returned to mothers during the experimental periods. Breeders were fed Wayne Lab Blocks.

Experimental protocol. Infant mice, which had been starved for 6 h, were inoculated orally with viable *V. cholerae* as described previously (3). The *V. cholerae* cells were steady-state labeled with $^{35}SO_4$ in a sulfate-limited modification of Richardson minimal AG medium (4, 8), washed three times to remove excess label, and diluted to approximately 10^8 CFU/ml

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in brain heart infusion (BHI) broth plus 0.01% Evans blue dye. The 0.05-ml input dose contained 4×10^6 to 7×10^6 CFU and 150,000 to 250,000 cpm.

Determination of specific activity. At 4 h postchallenge, infant mice were sacrificed by cervical dislocation, and the entire gut was removed. The proximal half of the intestines (upper bowel) was then cut away from the stomach and distal intestinal half, which were discarded. The upper bowel segments were homogenized in 1.0 ml of BHI broth, and 0.1-ml samples were removed for viable count and radioactivity determination. Viable counts were determined by plating appropriate dilutions in BHI broth onto meat extract agar in triplicate. Radioactivity was determined by liquid scintillation counting of perchloric acid-H_2O_2 digests (24) of 0.1-ml homogenate samples. Our procedure has been described in detail elsewhere (4). The specific activity was calculated from the formula: counts per minute/CFU. For the initial specific activity, the counts per minute per 0.05-ml dose was determined on duplicate samples, and the average was divided by the CFU per dose. At 4 h, specific activity was determined from the counts per minute per 0.1-ml homogenate sample divided by the CFU per 0.1-ml sample.

Results are expressed as a ratio of the value at 4 h compared with the initial value (i.e., $\text{parameter}_4/\text{parameter}_0$). The parameters were either CFU, counts per minute, or specific activities.

Localization of label. To determine whether label was associated with soluble or particulate matter, the homogenate (minus the two 0.1-ml samples) was suspended in 5.0 ml of a Krebs-Ringer buffer, KRT, as described by Jones and Freter (19), and centrifuged at $5,900 \times g$ for 20 min. A 0.1-ml sample of the supernatant was then counted as described. Results were expressed as a percentage of the total counts per minute in the supernatant.

Statistical analysis. *P* values were determined by the Wilcoxon rank test (30).

RESULTS AND DISCUSSION

Mechanisms of alteration in the specific activity. An analysis of the processes which may affect the specific activity of an infecting cell population during some time period postinoculation is presented in Table 1. Cases 1 and 2, which effect a decrease in specific activity, involve processes associated with a viable, metabolically active cell population. It should be noted that for case 2 to contribute to a decrease, the secreted components (primarily protein when ^{35}S is the label) must be removed from the environment of the viable cell population. Cases 3, 3′, and 4, which result in an increase in specific activity, involve mechanisms which would antagonize the establishment of successful infection. Aggregation (case 3′) of cells by specific antibody (or perhaps by other means) appears to restrict vibrios in their ability to associate effectively with the mucosa (5, 27). Cases 3 and 4 would occur in a bactericidal environment. Case 4 is included because one would expect the primary source of utilizable substrate to be lysed (i.e., nonviable) cells. Thus cases 3 and 4 would promote synergistically an increase in specific activity. It should also be noted that for the expected increase to be observed in cases 3 and 4, the nonviable cells or labeled components must remain in the environment. Case 3′ cannot be interpreted in terms of viability, as aggregates may consist of viable and nonviable cells, and case 5 indicates a bacteriostatic phenomenon.

This analysis is essentially ecological and defines those events which may affect the apparent specific activity value of a cell population in a given environment.

The chief advantage of using specific activity rather than viable counts for examination of survival and multiplication is that specific activity relates only to the infection site being considered and is thus not influenced by normal washout of bacteria. It should be realized that in vivo the situation is complex and that the changes which occur in the specific activity in a given time period represent the sum of all processes occurring in an essentially heterogeneous environment. However, analyses of changes in specific activity during the infective process indicate a predominance of certain of the possibilities outlined in Table 1.

Analysis of infective processes by determina-

TABLE 1. *Analysis of parameters which affect specific activity*

Change in specific activity	Case no.	Variable	Mechanism	Equation[a]
Decrease	1	CFU increase	Multiplication	$SA_t = SA_0/2^n$
	2	cpm decrease	Secretion	$SA_t = SA_0 - x$
Increase	3	CFU decrease	Killing	$SA_t = SA_0/(v/T)$
	3′		Aggregation	$SA_t = N(SA_0)$
	4	cpm increase	Utilization	$SA_t = SA_0 + y$
None	5	No change	Stasis	$SA_t = SA_0$

[a] Definition of terms: SA_t, specific activity at time t; SA_0, specific activity of challenge dose; *n*, number of generations; x, counts per minute (cpm) secreted/CFU during time t; v/T, fraction of total cells viable at time t; N, number of cells per aggregate; y, cpm taken up/CFU during time t.

tion of isotope distribution in the infecting viable population is not a new concept. Use of such procedures for studies of in vivo multiplication and survival potential has been suggested by several authors (7, 25). Furthermore, the techniques have been applied in studies of infections of respiratory (15) and intestinal (6, 9, 11) mucosal surfaces. However, a complete analysis of parameters which may affect isotope distribution (as shown in Table 1) has not been given. Rather, attention has been focused on cases 1 and 3 only.

In vivo changes in specific activity. Figure 1 shows the changes in specific activity and the viable organism recovery for strains CA401, VB12, and 569B from the upper bowel of infant mice at 4 h after oral inoculation. The results from four different experiments with CA401 and two experiments each with 569B and VB12 are given. It can be seen that viable CA401 was recovered in approximately 100-fold-greater numbers than were the other strains. This observation confirms our previous report of viable

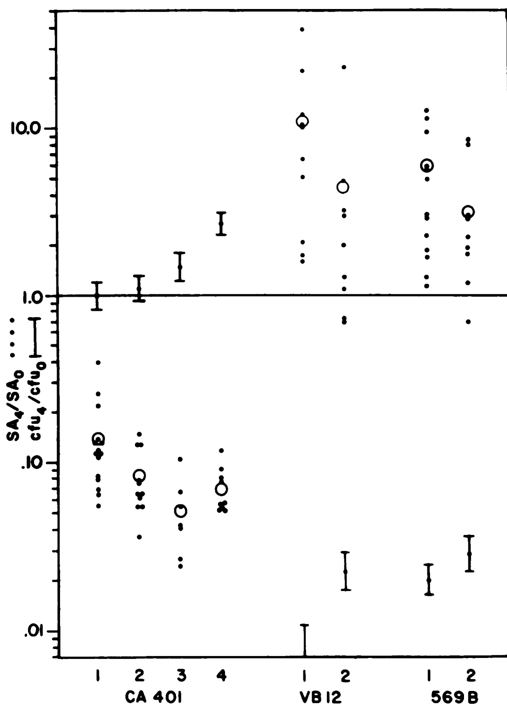

FIG. 1. *In vivo changes in apparent specific activity of ^{35}S-labeled V. cholerae in the upper bowel of infant mice at 4 h after oral inoculation with 4 × 10^6 to 7 × 10^6 CFU. Results are expressed as value at 4 h/value for the challenge dose. Symbols: ●, specific activity ratios of individual animals; ○, mean specific activity; I, mean CFU ratio ± 1 standard error of the mean.*

organism recovery with these strains (4). In addition, we noted that the distribution of the isotope relative to the viable cell population had been altered, as evidenced by changes in the specific activity. Whereas a decrease in specific activity was noted with CA401, an increase was seen for VB12 and 569B. The decreased specific activity value for CA401 (which indicates the presence of an active cell population) is consistent with the known infective potential of the strain. That is, the elicitation of disease in infant mice at this dose is necessarily related to the establishment of a viable infecting cell population.

Likewise, the increased specific activity observed with 569B and VB12 (which indicates decreased ability to establish infection) is consistent with their inability to induce disease in our model system. The results for the rough strain VB12 were expected, in view of the greater susceptibility of rough *V. cholerae* to host defense mechanisms (18, 28). The results found for 569B are compatible with the report by Knop and Rowley that there is reduced recovery of viable 569B from the surface of washed, adult mouse intestinal loops compared with another classical cholera strain (21). Furthermore, using radiotracer techniques, they demonstrated that this reduced recovery was probably due to greater killing of 569B and that the majority of isotope recovered was non-cell-associated and of very low molecular weight. This probably indicates that lysis of cells occurred.

We next examined the location of label with our strains in the infant mouse system.

Localization of radiolabel. The percentage of total counts in the upper bowel of infant mice at 4 h postchallenge which are recovered in the supernatant fraction after a 20-min 5,900 × *g* centrifugation is given in Table 2. A significantly greater amount of ^{35}S-labeled material is non-cell-associated for Formalin-killed CA401, VB12, and 569B ($P < 0.001$) than for CA401. In control experiments, we found homogenization of labeled vibrios with upper bowel segments from uninfected mice did not cause release of ^{35}S-labeled material from cells.

These observations plus the greatly reduced recovery of viable organisms with VB12 and 569B suggest that bactericidal and bacteriolytic mechanisms are operative in the infant mouse upper bowel. Florey also observed that a bacteriolytic process occurred on the surface of the small intestine (11). Strain CA401 is apparently able to circumvent host antibacterial mechanisms, perhaps by displaying resistance to killing or multiplying at a greater rate than killing occurs. However, the other experimental groups appeared to be susceptible to killing and lysis.

TABLE 2. *Label associated with nonparticulate material in upper bowel homogenate*

Test organism	No. of animals	CFU_4/CFU_0[a]	SA_4/SA_0[b]	% of cpm in supernatant	*P* compared with CA401
CA401	9	2.70 ± 0.44[c]	0.070	38.7 ± 2.4[c]	NA[d]
CA401 + Formalin[e]	9	NA	NA	93.0 ± 3.4	<0.001
VB12	9	0.024 ± 0.006	4.49	83.1 ± 5.3	<0.001
569B	9	0.030 ± 0.007	3.42	90.1 ± 4.5	<0.001

[a] CFU recovered at 4 h/CFU in the challenge dose.
[b] Specific activity at 4 h/specific activity of the challenge dose.
[c] Mean ± 1 standard error of the mean.
[d] NA, Not applicable.
[e] Treatment with 0.5% Formalin for 30 min.

This could be due to loss of cell surface resistance factors. Rough strains are known to be more sensitive to complement-mediated lysis (28) and may similarly be sensitive to other lytic factors. Alternatively, the organisms may be unable to express a phenotype in vivo which is essential in overcoming host defense processes. The observation that Formalin-killed CA401 cells which cannot effect phenotypic transformation seem susceptible to lysis supports the latter concept. However, it is possible that Formalin treatment alters some surface resistance factor.

The finding of soluble radioactive components also points out another essential feature of the infective process; that is, the soluble components are apparently not removed selectively from the infected environment. This is supported by previous studies (4) in which the kinetics of distribution of radiolabel for CA401 and Formalin-treated CA401 are similar in prediarrhea time periods. Because Formalin-treated CA401 inocula are recovered almost entirely as soluble material at 4 h, it seems that removal of non-cell-associated and cell-associated labeled materials occurs at the same rate.

This observation influences interpretation of changes in specific activity. Thus, a decrease due to secretion (which requires removal of product) will not be noted; rather, a decrease may be primarily attibuted to multiplication. However, increases due to killing and lysis or utilization of soluble labeled materials may be noted. Because of possible utilization phenomena, one should be aware that calculation of percent killing (Table 1, case 3) may give a falsely elevated value.

Although secretion may not actually contribute to specific activity changes in our system, it is notable that CA401, which appears to establish a multiplying cell population in the upper bowel, yielded a recovery of 38.7 ± 2.4% of the label in the supernatant fraction. This label may represent secreted products. Cells similarly labeled and suspended in vitro in a rich medium (BHI broth) for 4 h at 33°C (the approximate infant mouse body temperature [2]) release 24% of their total label into supernatant; this is likely to reflect secretion. However, in vivo, it is also possible that some soluble label comes from lysed cells. Furthermore, it should be realized that binding of soluble products to particulate fractions (e.g., toxin to mucins [29] or epithelial cell membranes [17] or enzymes to particulate substrate carriers) occurs. Thus, not all the non-vibrio-associated label would be recovered in the supernatant fraction.

These studies provided insight into the possible contributions of cases 2, 3, and 4 (Table 1) to changes in specific activity seen in vivo. The next experiments attempted to assess the role of the other processes outlined in Table 1.

Role of aggregation: case 3′. To examine whether formation of aggregates contributed to the 4-h specific activity values found, Gram stains were done on the upper bowel homogenates of nine animals per strain. The homogenates were found to have a relatively even dispersion of materials. Vibrios were easily recognizable as gram-negative curved rods. For CA401-infected animals, organisms were frequently observed as single, isolated cells. No aggregates were seen. However, occasional S-shaped dividing forms were observed. For VB12 and 569B, as expected from the label localization studies, intact organisms were infrequently observed. No aggregates were observed, and no S forms were seen.

Thus, although aggregation of organisms as observed by Koch (26) or mediated by specific antibody (5, 27) occurs in vivo in the intact intestine, our homogenization procedure apparently disrupted such aggregates. Therefore, we believe that case 3′ is not operative as a mechanism contributing to an increase in specific activity in our experiments.

Multiplicative potential of CA401. The preceding sections have indicated that VB12 and 569B are both unable to survive host defense mechanisms. However, CA401 is able to survive, multiply, and establish an infection in the infant

mouse upper bowel. We were interested in comparing the multiplicative potential of CA401 in vivo and in vitro. Results are given in Table 3. The results were expressed as the apparent number of generations (n) which occurred in 4 h under the conditions described. In the calculations, the assumption was made that secretion is not a contributing factor to a decrease in specific activity. In vitro, n was the same whether determined from viable counts or specific activities because the only variable in specific activity was CFU; i.e., counts per minute were constant. In vivo, n based on viable counts was 2.3-fold lower than n based on specific activity. This was an expected finding, as washout of progeny must occur, thus depressing the value determined from CFU. However, the in vivo n value based on specific activity was similar to the in vitro n and was actually slightly larger (13%). Washout of progeny would not affect the calculation of n from specific activity, because one is concerned only with the label distribution in the recoverable infecting population. However, washout of non-cell-associated products (which would decrease the specific activity) would falsely elevate n compared with the true n. Because soluble material appears not to be removed at an appreciably greater rate than particulate material, the theoretical n probably provides an accurate indication of multiplicative potential. Furthermore, the in vivo multiplication pattern is very similar to in vitro growth in rich media at the approximate mouse body temperature.

Evaluation of infective potential by using specific activity. We feel that analysis of the changes in specific activity which occur early in infection provides a relevant appraisal of the ability of a strain to establish an infection (i.e., survive and multiply) in the susceptible host environment. Any infection is characterized by two concurrent processes: colonization and elimination. Colonization refers to the establishment of a metabolically active population at the site of infection. Elimination refers to the processes which prevent the establishment of the infecting population. Therefore, for colonization to occur, elimination processes must be circumvented. As outlined in Table 1, whereas a decrease in specific activity indicates the presence of a metabolically active, multiplying cell population (i.e., colonization), an increase indicates a predominance of processes preventing colonization (i.e., elimination). No change indicates either survival but no multiplication or an even balance between colonization and elimination. Our data indicate that in our model system the major mechanisms involved in changes in specific activity are defined by cases 1 and 3 and possibly by cases 4 and 5.

TABLE 3. *Number of generations of ^{35}S-labeled CA401 in vivo and in vitro in 4 h*

Condition[a]	Calculated n[b]	Theoretical n[c]
In vitro (stationary BHI broth, 33°C)	3.0	3.0
In vivo (upper bowel of infant mice challenged orally with 0.05 ml)[d]	1.5	3.4

[a] Cells were labeled with $^{35}SO_4$ as described in the text and suspended to a density of 10^8 CFU/ml in BHI broth.

[b] n is the number of generations by the equation: 2^n = CFU at 4 h/CFU at zero time.

[c] n is the number of generations by the equation: 2^n = specific activity at zero time/specific activity at 4 h.

[d] Mean from 42 animals.

Based on the results reported in this paper, we have defined the infective potential of these strains in terms of the alterations in specific activity which occur in the upper bowel of infant mice at 4 h after oral inoculation. The equation is: infective potential = −log (specific activity at 4 h/specific activity of inoculum). The infective potentials for CA401, 569B, and VB12 are shown in Table 4. A positive number (CA401) indicates colonization, a negative number (VB12, 569B) indicates elimination, and zero would indicate no change in specific activity. The degree of colonization or elimination is related to the absolute value. Thus, VB12 was eliminated slightly better than 569B.

It should be noted that the parameter of infective potential could be used to evaluate the virulence of other organisms with any radiolabel and at different sites of infection. However, appropriate controls must be performed to select significant times and sites for analysis and the contribution of each case in Table 1 should be evaluated.

We are currently using the parameter of infective potential with a number of phenotypically distinct mutants of CA401 which do not evoke diarrhea to assess whether their inability to elicit disease is due to decreased colonization ability or increased susceptibility to elimination.

TABLE 4. *Infective potential of V. cholerae strains for infant mouse upper bowel*

Strain	Mean infective potential[a]	No. of animals
CA401	1.0	42
569B	−0.6	22
VB12	−0.9	19

[a] Calculated from the equation: infective potential = −log (specific activity at 4 h/specific activity of inoculum).

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